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Glucocorticoids Inhibit Apoptosis during Fibrosarcoma Development by Transcriptionally Activating Bcl-x_L*

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Glucocorticoids influence many physiological processes, and in particular apoptosis, often with opposite effects depending on the cell type examined. We found that during fibrosarcoma development there is a strong increase in apoptosis at the tumor stage, which is repressed by dexamethasone to levels observed in normal fibroblasts. The anti-apoptotic Bcl-2 family protein Bcl-x_L is induced by dexamethasone at the transcriptional level at all stages of fibrosarcoma development. The ligand-activated glucocorticoid receptor (GR) activates the Bcl-x promoter in transient transfection experiments, and GR binds to specific Bcl-x promoter sequences *in vitro* and *in vivo*. Furthermore, a GR antagonist abolishes this effect, indicating that Bcl-x_L induction is mediated by GR. Importantly, exogenous Bcl-x_L inhibits apoptosis and caspase-3 activity in fibrosarcoma cells to levels found in dexamethasone-treated fibrosarcoma cells. We conclude that Bcl-x_L is a key target mediating the anti-apoptotic effects of glucocorticoids during fibrosarcoma development. These observations provide further understanding of the molecular basis of glucocorticoid regulation of cell death during tumorigenesis.

Glucocorticoids exert different effects on apoptosis and cell growth depending on the tissues examined. In some cell types, for example thymocytes and some leukemia cell lines, treatment with glucocorticoids induces apoptosis (1). This has led to their common use as chemotherapeutic agents in lymphomas and leukemias (2). In contrast, glucocorticoids have been reported to inhibit apoptosis in a number of other cell types, including glioma and astrocytoma cell lines (3), fibroblasts (4), hepatoma cells (5), gastric cancer cell lines (6), and mammary epithelial cells (7, 8).

The glucocorticoid receptor (GR)[1] belongs to a superfamily of transcription factors that includes receptors for steroid and thyroid hormones, retinoic acid, and vitamin D_3 (9). GR is normally localized in the cytoplasm in a non-active state in a complex that includes Hsp90. Upon hormone binding, GR changes conformation and migrates to the nucleus, where it induces or represses transcription by binding to specific DNA sequences on target genes (10).

Apoptosis, triggered by a variety of intra- and extracellular signals, is important for normal development, to maintain tissue homeostasis, and as a defense strategy against the emergence of cancer (11, 12). The apoptotic program is executed by a family of cysteine proteases called caspases, which are activated by proteolytic cleavage (13, 14). Once activated, effector caspases cleave a variety of cellular substrates including structural components, regulatory proteins, and other caspases, resulting in the orchestrated collapse of the cell characteristic to apoptosis.

Bcl-2 family proteins play critical roles in the control of apoptosis. Two major groups of Bcl-2 family proteins exist; the pro-survival members, including Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, etc., and the pro-apoptotic members, including Bax, Bak, Bok, etc. (see Ref. 15 for review). The ratio between these two groups of family members determines whether a cell will live or die. Downstream of this checkpoint lie the caspase pathway and mitochondria dysfunction, major execution events that lead to irreversible cell death (16). Alterations in the expression of anti-apoptotic members such as Bcl-2 and Bcl-x_L have been implicated in tumorigenesis in both clinical cases and transgenic models (17). In addition, Bcl-2 members are also important determinants of anticancer drug sensitivity (18).

The conversion of a normal cell to a neoplastic one occurs in multiple steps (19), and one approach to studying this process has been to employ transgenic mice (20). Mice carrying the bovine papillomavirus type I genome develop dermal fibrosarcomas in a process that involves distinct proliferative stages. These are the normal fibroblasts (NF), and then two histological grades of hyperplasia, mild fibromatosis (MF) and aggressive fibromatosis (AF). Finally, at lower frequency, dermal fibrosarcomas (FS) develop (21). The first molecular distinction between the AF and the FS cells to be identified was a dramatic increase in ligand-dependent GR transcriptional activity in FS cells (22). This increase does not result from changes in the intracellular levels of GR, hormone-dependent nuclear translocation, or specific DNA binding activity, all of which are unaltered throughout the progression. Moreover, analysis of the tumors formed in mice upon inoculation of AF or FS cells indicates a direct correlation between GR transcriptional activity and tumorigenic potential (22).

To understand cancer progression it is important to determine the mechanisms by which signaling proteins influence proliferation and apoptosis at different stages of the tumorigenic process. Here we have examined the effects of dexamethasone on apoptosis in the multistep tumorigenic pathway

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[1] The abbreviations used are: GR, glucocorticoid receptor; ChIP, chromatin immunoprecipitation; NF, normal fibroblast; MF, mild fibromatosis; AF, aggressive fibromatosis; FS, fibrosarcoma; GRE, glucocorticoid response element; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; bFGF, basic fibroblast growth factor; TK, thymidine kinase.

of fibrosarcoma development. Our observations point to Bcl-x_L as a key GR target mediating the inhibition of apoptosis during fibrosarcoma progression.

EXPERIMENTAL PROCEDURES

DNA Plasmid Constructions—The plasmid containing 3.2 kb (−3292 to −94) of the 5′-region of the murine Bcl-x promoter linked to the luciferase reporter gene (23) was a kind gift of Gabriel Nuñez (Ann Arbor, MI), and was designated Bcl-x(3.2). Serial promoter deletion fragments were cloned into pGL2-basic (Promega) as follows: Bcl-x(2.8) (−2829 to −94) as a *Bgl*II and *Hin*dIII fragment; Bcl-x(0.6) (−679 to −94) by *Sma*I and partial *Hin*dIII digestion; Bcl-x(0.2) (−299 to −94) as an *Xmn*I and *Hin*dIII fragment, and Bcl-x(0.1) (−199 to 094) as a *Kpn*I and *Hin*dIII 110-bp fragment.

Fragments containing the putative GREs from the Bcl-x promoter were cloned into a TK_{109}-luciferase reporter construct: RE1 contains sequences of the murine Bcl-x promoter from −2963 to −2268. This fragment was amplified by PCR using Bcl-x(3.2) as template and the oligonucleotides A (5′-GTTTCCCAAAAGGATCCAATG-3′) and C (5′-AAATGCGGATCCTGACTGACTG-3′). The product was digested with *Bam*HI and inserted into TK_{109}-luciferase. RE2 contains sequences from −2963 to −2826, which were amplified by PCR using the oligonucleotides A and D (5′-CTGGTTATGTAGCTGTGGGCTGCC-3′). The PCR product was digested with *Bam*HI and *Bgl*II. RE3 contains sequences from −2349 to −2268 of the Bcl-x promoter, which were amplified by PCR using the oligonucleotides B (5′-AAGTGGATCCTC-CTATGCTAC-3′) and C. The product was digested with *Bam*HI.

The putative GREs from the Bcl-x promoter were mutated using the following oligonucleotides (nucleotides that were changed are underlined) and their reverse complements: P1 (5′-CTCTGTGGCCAACAGT-CCATTCTGCGAAAGACGGGAAAGTTGC-3′), P2 (5′-GCTGTGCAGA-AGACCAGCTTTTTCCTGAGGCCATGTTATCC-CACAGCCAGG-3′), P3 (5′-GCTACATAGATTGAGGCCAGACTCGGCTGAAAAACTG-3′). The QuikChange XL site-directed mutagenesis kit (Stratagene) was used following the instructions of the manufacturer. All mutations were confirmed by DNA sequencing.

The fragment of murine Bcl-x_L cDNA encompassing the coding region was amplified by PCR from the total cDNA of NF cells using HiFi polymerase (Roche Applied Science). This fragment was cloned into pIRES2-eGFP (Clontech). Absence of mutations within the insert was confirmed by sequencing in all cases.

Cell Culture and Transient Transfections—Cultures were established from skin and tumor tissues and maintained as reported previously (22). Several cultures were tested from each stage of the tumorigenic process: normal fibroblasts (23784, 40950); mild fibromatosis (14249, 39614, 27877); aggressive fibromatosis (BPV3, BPV7, BPV21); fibrosarcomas (BPV1, BPV22, BPV2, BPV11). For experiments involving steroid hormone treatments, cells were maintained in medium containing charcoal-stripped serum (24). COS-7 cells were also maintained as above. Cell viability was determined by trypan blue dye exclusion (25).

Cells were transiently transfected using the DEAE-dextran method and the β-galactosidase expression vector 6RZ as internal control for efficiency of transfection as described previously (22). After exposure to the DNA/DEAE-dextran mixture, the cells were incubated for 36 h in medium containing charcoal-stripped serum with or without 100 nM dexamethasone (Sigma). The GR antagonist RU 40555 was kindly provided by Roussel-Uclaf (Romainville, France). Luciferase activity was measured according to the instructions of the manufacturer (Promega).

RNA Isolation and Northern Blot Analysis—Total RNA was isolated using the SV Total RNA isolation system (Promega) according to the instructions of the manufacturer. Northern blot analysis was performed using conventional techniques. The probe encompassing 725 bp of the Bcl-x_L cDNA was labeled using a Random Primed DNA labeling kit (Roche Applied Science) following the instructions of the manufacturer. Finally, the membrane was exposed to x-ray film (Kodak X-Omat AR).

Preparation and Analysis of Cell Extracts—Cells were harvested by centrifugation in 40 mM Tris-HCl, pH 7.8, 10 mM EDTA, and 150 mM NaCl, washed in ice-cold phosphate-buffered saline, and re-pelleted and frozen in liquid nitrogen. Cells were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 10 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin/leupeptin/pepstatin A), incubated on ice for 25 min, and centrifuged for 20 min at 14,000 rpm. Equal amounts of extract protein (30 μg) were analyzed by immunoblot analysis as described previously (22). Anti-Bcl-2 (Oncogene), anti-Bcl-$x_{L/S}$ (Transduction Laboratories), anti-Bax (Zymed Laboratories Inc.), anti-β-actin (Sigma), and anti-tubulin (Sigma) were used as primary antibodies, followed by incubation with the corresponding secondary antibody (horseradish peroxidase-conjugated; Bio-Rad). Protein-antibody complexes were visualized by an enhanced chemiluminescence immunoblotting detection system (Amersham Biosciences).

TABLE I
Stages of murine fibrosarcoma development

The integrated bovine papillomavirus type-1 (BDV-1) transgene present in NF cells is excised and transcribed in MF, AF, and FS cells. However, E5 and E6 oncogene products are detectable only in the latter two cell types (21). All cells forming tumors *in vivo* exhibit FS phenotype (22). Increased activity of cellular AP-1 components JunB and c-Jun is apparent in AF and FS cells (22, 50), but dramatically increased GR activity is unique to the tumor cells (22).

	NF	MF	AF	FS
BPV-1 RNA	−	+	+	+
E5, E6	−	−	+	+
Tumor	−	−	−	+
JunB, c-Jun	+	+	+++	++++
GR activity	+	+	++	+++++

Gel Retardation Assays—Analysis of DNA-protein interactions was performed as described (22). The following oligonucleotides were used: consensus GRE-TAT (22), P1 (5′-TGGCCAACAGTACATTCTGTGAAA-GAC-3′), P2 (5′-TGTGCAGAAGAACAGCTTTTTCC-TGAGGCCATGT-TGTCCCACAG-3′), and P3 (5′-ATAGATTGAGGACAGACTGGGCTG-AAA-3′); nonspecific, N (5′-AGGATAACGGAGGCTGGGTAGGTGCAC-3′). To assess specificity of DNA binding, 50-fold molar excess of unlabeled oligonucleotide (consensus GRE, specific (S) or same as labeled oligonucleotide) or 200-fold molar excess of a nonspecific oligonucleotide (N) was added to the reaction prior to addition of the labeled probe.

Caspase Enzyme Activity Assay—Caspase assays were performed using a protocol based on the QuantiZyme™ assay system (Biomol). Cells were lysed in buffer Q (50 mM HEPES, pH 7.4, 0.1% CHAPS, 0.1 mM EDTA, 0.1 mM dithiothreitol) for 30 min on ice followed by centrifugation at 14,000 rpm for 10 min at 4 °C. Equal amounts of protein (30 μg) were added in triplicate to wells in a 96-well plate and equilibrated to 37 °C for 15 min after the addition of assay buffer (50 mM HEPES pH 7.4, 0.1% CHAPS, 1 mM EDTA, 10 mM dithiothreitol, 100 mM NaCl, 10% glycerol). Fluorogenic peptide substrates selective for caspase-1 (YVAD) or caspase-3 (DEVD) (final concentration 1 mM) were added to each well and incubated at 37 °C for 6 h. For controls, the caspase inhibitors Ac-YVAD-CHO or Ac-DEVD-CHO, respectively, were used at a final concentration of 0.5 μM. Absorbance of the samples at 405 nm was measured using a colorimetric plate reader (MRX) immediately after addition of the substrate and after 6 h. The change in absorbance of each sample (minus the inhibitor control) over the 6-h period was taken as the specific caspase activity.

Chromatin Immunoprecipitation (ChIP) Assay—Cells were maintained in 10-cm dishes in medium containing charcoal-stripped serum for at least 16 h and treated with or without 100 nM dexamethasone for 1 h. The ChIP assay was performed as described previously (26) except that the final samples were resuspended in 30 μl of water, and 2–4 μl of each sample was used for PCR amplification (30 cycles) with the r*Taq* DNA polymerase (TaKaRa). The antibody used against GR was sc-1002X, and sc-751 against cyclin A was used as a negative control (both from Santa Cruz Biotechnology). The primers for the PCR were 5′-CCCAAAAGGATAGAATGAACTCTG-3′ and 5′-CTGGTTATGTAGCT-GTGGGCTGCC-3′, which amplify a 247-bp fragment corresponding to murine Bcl-x sequence −2976 to −2730 from the transcription start site (GenBank™ accession number AF 088904). As negative control the primers 5′-TGGGCTGGCTCCCTGGTCAG-3′ and 5′-CCTCGGTCACG-GATCTTAGGC-3′, which amplify a 112-bp fragment corresponding to murine Bak sequence −960 to −848 (GenBank™ accession number Y 13232), were included in the same PCR reactions.

RESULTS

Cell Death Is Reduced by Dexamethasone Treatment—Cell proliferation increases during fibrosarcoma development *in vivo* (27) and in cultured cells (21), and it is inhibited by dexamethasone, a GR agonist (53). We examined the effects of glucocorticoids on cell death during fibrosarcoma progression. We used low-passage primary cell lines representative of the four stages in this multistep pathway: NF, MF, AF, and FS

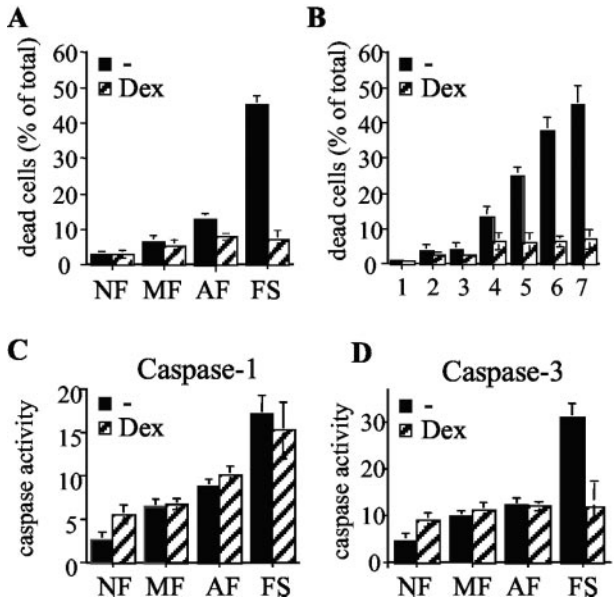

FIG. 1. **Effects of dexamethasone (*Dex*) on cell death during fibrosarcoma development.** *A*, the number of dead cells as a percentage of total population of NF, MF, AF, and FS cells. Cells from each stage of fibrosarcoma progression were plated at 2×10^4 cells/well 24 h prior to treatment with (*solid bars*) or without (*shaded bars*) 100 nM dexamethasone. Values represent the mean percentage of total cells counted in triplicate, within one representative experiment (repeated three times). *Error bars* represent standard deviation in all figures. *B*, dead FS cells on each day of culture. *C* and *D*, caspase-1 and caspase-3 enzyme activities in NF, MF, AF, and FS cells. Duplicate nonconfluent dishes of cells were untreated or treated with dexamethasone for 24 h. Values represent the average of three independent experiments. Caspase activity is in absorbance units ($\times 10^{-2}$).

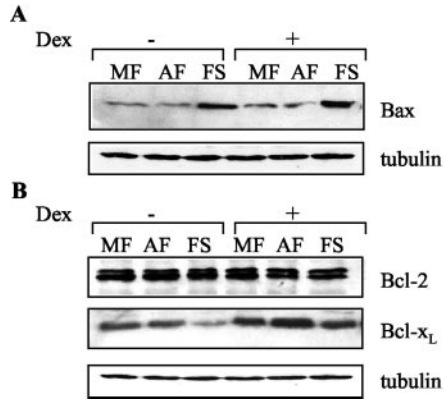

FIG. 2. **Expression of endogenous Bcl-2 family proteins in MF, AF, and FS cells.** Immunoblot analysis of pro-apoptotic (Bax) (*A*) and anti-apoptotic (Bcl-2, Bcl-x_L) (*B*) from MF, AF, and FS cells untreated (−) or treated (+) for 24 h with 100 nM dexamethasone (*Dex*). Cell extracts were probed with antibodies against various members of the Bcl-2 family (for details see "Experimental Procedures").

cells (see Table I). Cells from each stage were cultured in the absence and presence of dexamethasone, and cell growth was observed for 7 days at 24-h intervals. In the absence of hormone, cells from the earlier stages of the progression proliferated at a lower rate, as reported previously, whereas FS cells increased rapidly in number (21). Cells cultured in the presence of dexamethasone appeared flattened (data not shown) and were fewer in number. Trypan blue exclusion was used as a marker for viability of the cells. The number of dead cells, calculated as a percentage of total population at each of the progression stages (Fig. 1*A*, dead cells at day 7), indicated that the extent of cell death was higher in the tumor cells than in the primary cells and that dexamethasone reduced the number of dead cells, particularly among FS cells. Fig. 1*B* shows the proportion of dead FS cells on each day of culture. Thus dexamethasone exerts a protective effect on FS cells.

To assess whether the observed cell death reflected apoptosis, we measured the caspase activity in cells from all stages of fibrosarcoma development. Caspase-3 has been shown to be one of the key effectors of apoptotic cell death (28), whereas caspase-1 is involved in inflammatory responses and also in some forms of apoptosis (29). In the absence of hormone there was an increase in both caspase-1 and caspase-3 activity during tumor progression ($p < 0.01$), particularly at the fibrosarcoma stage (Fig. 1, *C* and *D*). Upon dexamethasone treatment, caspase-3 activity was ~50% reduced in FS cells ($p < 0.005$), suggesting an inhibition of apoptosis. In contrast, caspase-1 activity was not affected by the presence of hormone. In addition, when we examined the extent of cell death by DNA fragmentation analysis, we detected a high degree of DNA degradation in the FS cells, which was abolished by hormone (data not shown). These results confirm that there is a strong increase in apoptosis at the tumor stage of fibrosarcoma development and that this is reduced by dexamethasone.

Changes in the Expression of Bcl-2 Family Proteins during Fibrosarcoma Development—Bax appears to be one of the major pro-apoptotic Bcl-2 proteins that act as death effectors in fibroblasts, and Bcl-2 and Bcl-x_L serve as inhibitors of Bax (30). Furthermore, Bcl-x_L has been found to be regulated by dexamethasone in some cell types (8, 31). Therefore, to investigate changes in the level of expression of Bcl-2 family proteins that might contribute to the regulation of apoptosis during fibrosarcoma development, we examined the expression levels of these proteins in the absence and presence of dexamethasone by immunoblot analysis (Fig. 2). NF and MF cells expressed similar levels of the Bcl-2 family members examined hence only MF results are shown in this figure. Expression of the pro-apoptotic protein Bax was elevated in FS cells, but its protein levels were not affected by dexamethasone (Fig. 2*A*). The increased expression of Bax protein correlates with increased apoptosis in FS cells. However, the hormone-dependent inhibition of apoptosis we observed in FS cells does not correlate with Bax levels.

Analysis of the expression of anti-apoptotic proteins (Fig. 2*B*) showed that Bcl-2 migrated as a doublet and remained constant in all cell types throughout tumor progression. In contrast, Bcl-x_L expression clearly decreased during fibrosarcoma progression. However, in the presence of dexamethasone its expression was strongly induced, correlating with the much reduced level of apoptosis in dexamethasone-treated FS cells (Fig. 1).

The combination of changes in the expression levels of apoptotic proteins in FS cells, together with elevated caspase-3 activity, correlates with the increase in apoptosis at the tumor stage, suggesting that Bcl-2 proteins play a role in regulating apoptosis during fibrosarcoma development. Furthermore, Bcl-x_L induction in response to hormone in FS cells makes Bcl-x_L a candidate for mediating the reduction in apoptosis at the tumor stage.

Bcl-x_L Expression Is Induced by Dexamethasone—A more detailed analysis of expression of Bcl-x_L protein during fibrosarcoma progression showed a clear increase in the fold induction of the protein by dexamethasone as fibrosarcoma progresses (Fig. 3*A*). This pattern of hormone-dependent induction of expression correlates with the transition in GR transactivation activity observed during fibrosarcoma progression (22) and suggests that GR might be involved directly in Bcl-x_L regulation. The analysis presented involved representative cell cultures from the four stages of the pathway (NF 40950, MF

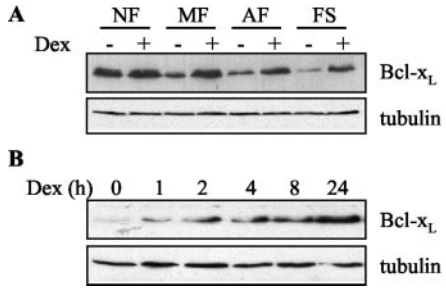

FIG. 3. **Bcl-x_L protein expression during fibrosarcoma progression following dexamethasone (*Dex*) treatment.** *A*, immunoblot analysis of Bcl-x_L protein from NF, MF, AF, and FS cells untreated (−) or treated (+) with 100 nM dexamethasone for 24 h. Cell extracts from each stage of fibrosarcoma progression were probed with a rabbit antibody against Bcl-x_L. *B*, time course of Bcl-x_L protein induction in FS cells in response to dexamethasone for the indicated amount of time (0–24 h). All cells were harvested 48 h after plating and were growing exponentially.

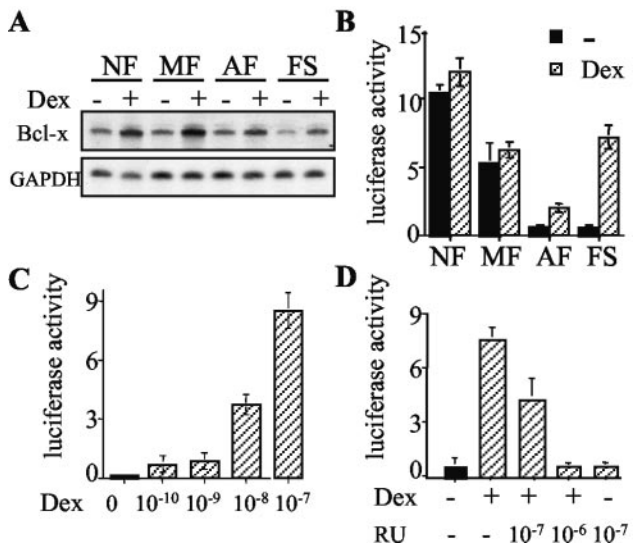

FIG. 4. **Regulation of Bcl-x transcription by dexamethasone (*Dex*).** *A*, cells from the various stages of fibrosarcoma progression were untreated (−) or treated (+) with 100 nM dexamethasone for 4 h. Northern blot analysis of Bcl-x mRNA levels using a 725-bp fragment from the Bcl-x_L cDNA (IMAGE 1395857) as probe. The membrane was also probed for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). *B*, expression of the Bcl-x(3.2) luciferase reporter construct in NF, MF, AF, and FS cells. The cells were either untreated (*black bars*) or treated for 36 h with 100 nM dexamethasone (*shaded bars*). Transcriptional activity of endogenous GR was analyzed. In all transfection experiments, luciferase activity was normalized to the β-galactosidase activity of a co-transfected reporter. The values presented are the mean of at least five different experiments. Normalized luciferase activity units are $\times 10^3$ in all experiments. *C* and *D*, expression of the Bcl-x(3.2) reporter in FS cells in response to increasing concentrations of dexamethasone (*C*) or to various hormone treatments, 100 nM dexamethasone, and/or the GR antagonist RU 40555 (*RU*) (*D*).

14249, AF BPV3, FS BPV1). However, we assayed the expression of Bcl-x_L in two or more independent cell lines from each stage (see "Experimental Procedures") and found that all lines derived from a particular stage displayed consistent results. To determine the time course of Bcl-x_L induction in FS cells, we examined protein levels at various times after dexamethasone treatment (Fig. 3*B*). Induction of Bcl-x_L protein could be detected as early as 2–4 h following exposure to the hormone and remained elevated for 24 h.

Bcl-x Transcription Is Induced by Dexamethasone—The rapid increase in the level of Bcl-x_L protein by dexamethasone in FS cells suggested that this induction occurred at the transcriptional level. Therefore, we examined Bcl-x mRNA expression after exposure to dexamethasone in all stages of fibrosarcoma development. Northern blot analysis revealed a major band of ~3-kb mRNA in all cell stages (Fig. 4*A*), as reported previously (32). In the absence of hormone, the expression level of the Bcl-x transcript was reduced during tumor development, similar to the observed decrease in protein levels. Bcl-x mRNA was increased by the presence of dexamethasone.

To investigate the regulation of Bcl-x promoter activity by dexamethasone, we transiently transfected a luciferase reporter construct containing a 3.2-kb genomic fragment of the murine Bcl-x promoter, Bcl-x(3.2), together with a β-galactosidase expression plasmid to normalize for transfection efficiency, into cells from each stage of fibrosarcoma progression. In the absence of dexamethasone, the activity of the promoter decreased from NF to FS cells (Fig. 4*B*) suggesting that Bcl-x_L protein expression decreases during tumor development in the absence of dexamethasone, primarily as a result of reduced Bcl-x transcription. Upon dexamethasone treatment, promoter activity was strongly increased in FS cells, indicating that glucocorticoids induce Bcl-x transcription (Fig. 4*B*). Various concentrations of dexamethasone were tested, and concentrations as low as 10^{-10} M significantly increased Bcl-x promoter activity ($p < 0.005$) (Fig. 4*C*).

To assess whether the induction of Bcl-x transcription by dexamethasone is dependent on GR, we tested the effect of the GR antagonist RU 40555 on transcriptional regulation of the Bcl-x promoter. In FS cells, dexamethasone-dependent Bcl-x promoter activity was inhibited by RU 40555 in a dose-dependent manner (Fig. 4*D*), whereas it did not have any effect by itself, demonstrating a requirement for transcriptionally active GR to induce Bcl-x expression.

GRE-like Sequences in the Bcl-x Promoter Mediate GR Activation—To understand further the transcriptional regulation of Bcl-x expression by dexamethasone, we searched for potential sequences within the Bcl-x promoter that confer hormone responsiveness. A series of luciferase vectors were constructed containing 5′ fragments of the 3.2-kb region of the Bcl-x promoter (Fig. 5*A*) and were transiently transfected into FS cells. Analysis of these fragments showed a clear reduction in the ability of the promoter to respond to dexamethasone, from around 8–10-fold induction using the 3.2-kb fragment to a small but significant ($p < 0.005$) induction using the most proximal region (Fig. 5*B*). The highest induction from the latter was obtained using Bcl-x(0.1), suggesting that this region (−199 to −94) contains a proximal weak glucocorticoid response element (GRE). Although this fragment does not contain classical consensus GRE sequences, it does contain putative binding sites for factors known to interact with GR, such as C/EBP (33) and Oct-1 (34). However, it appears that the major hormone-inducible promoter sequences are located within −3292 to −679.

To investigate further the glucocorticoid-responsive sequences within the Bcl-x promoter, the murine genomic Bcl-x sequence (GenBank™ accession number AF 088904) was screened for potential GR binding sites by computer analysis (MatInspector program, San Diego Workbench). Three candidate sequences, P1–P3 (with P2 containing two adjacent sites), were identified with some homology to the consensus GRE sequence GGTACANNNTGTTCT (35) in the sequence between −2944 and −2316 (Fig. 5*C*). To test whether these putative GREs (or GRE-like elements) are sufficient to mediate hormone induction, three fragments containing these sequences (RE1, −2963 to −2268, RE2, −2963 to −2826 and RE3, −2349 to −2268, Fig. 5*C*) were cloned into a luciferase reporter plasmid driven by a TK_{109} promoter (TK_{109}-luciferase). All three reporter plasmids displayed a clear induction of transcription in response to dexamethasone treatment (Fig. 5*D*). The largest

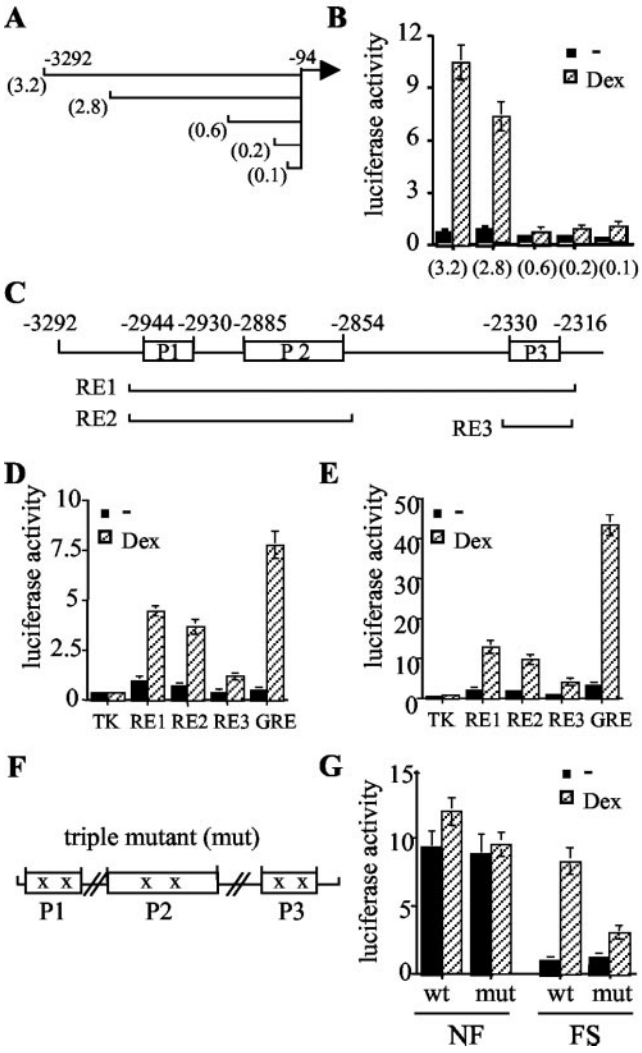

FIG. 5. **Bcl-x promoter analysis.** *A*, Bcl-x promoter fragments. *Dex*, dexamethasone. *B*, activity of the Bcl-x luciferase constructs shown in *A* in FS cells. The values presented are the mean of four different transfection experiments done in duplicates. Cells were untreated (*black bars*) or treated for 36 h with 100 nM dexamethasone (*shaded bars*). *C*, putative dexamethasone-responsive sequences within the Bcl-x promoter. *D* and *E*, activity of the Bcl-x constructs shown in *C* in FS cells (*D*) and in COS-7 cells (*E*) with conditions as described in *B*. *F*, triple mutant (*mut*) reporter resulting from the mutation of 2 bp in each of the three GREs (*P1*, *P2*, and *P3*) in the Bcl-x(3.2) reporter construct. *G*, transcriptional activity of the wild type (*wt*) and triple mutant Bcl-x(3.2) reporters (*mut*) with conditions as described in *B*.

fragment, RE1, which encompasses all four putative GREs, displayed the strongest transcriptional activity, followed by RE2 and RE3. Importantly, these sequences also conferred hormone responsiveness when co-transfected with a GR expression vector into COS-7 cells (Fig. 5*E*) although to a lesser extent than a reporter construct driven by a consensus GRE (TAT1, one copy of the GRE from the tyrosine aminotransferase gene promoter in front of the TK_{109} promoter). A construct containing an unrelated sequence (consensus thyroid response element, TK) upstream of the TK_{109}-luciferase did not respond to dexamethasone. These results confirm that dexamethasone-responsive sequences of the Bcl-x promoter lie within (−2944 to −2826) and (−2330 to −2268).

Finally, two point mutations in positions known to be important for GR-dependent transcription in the context of a consensus GRE (35) were introduced in each of the three putative GREs, and their effect on dexamethasone-dependent induction of the Bcl-x promoter was determined in transient transfection assays. Single and double combinations of these mutations decreased Bcl-x inducibility by dexamethasone in an additive fashion (data not shown), whereas introduction of two point mutations in each of the putative GREs (Fig. 5*F*) resulted in strong reduction of dexamethasone-dependent transcriptional activity in FS cells (Fig. 5*G*). In NF cells the response to dexamethasone is much weaker, but the inhibitory effect of mutating the GREs was still detectable. Although mutation of these binding sites does not abolish dexamethasone-dependent transcriptional activation of the Bcl-x promoter in FS cells totally, this was not surprising, because we know that the Bcl-x promoter contains other weak proximal putative GREs (Fig. 5*B*), and it is still conceivable that there are further weak putative GREs that remain unidentified. However, our results suggest that the integrity of the three GREs we have identified is required for GR-dependent activation of transcription.

GR Binds to the Bcl-x Promoter in Vitro and in Vivo—The ability of GR to bind to the identified GRE sequences was examined by gel mobility shift assays. We incubated nuclear extracts from hormone-treated FS cells in the presence of labeled oligonucleotides containing either one of the putative GREs (Fig. 5*C*, *P1–3*). A retarded complex was observed using P1 (Fig. 6*A*, *lane b*), P2 (*lane g*), and P3 (*lane j*) that migrated at the same position as that originated by binding of FS cell extracts to the consensus GRE-TAT (*lane m*). This complex has been shown to represent GR protein specifically bound to the GRE sequences (22). Incubation with 50-fold excess of the corresponding unlabeled oligonucleotides abolished binding to these sequences (Fig. 6*A*, *lanes c*, *f*, *k*, and *n*), whereas 200-fold excess of nonspecific unlabeled oligonucleotide had no effect (*lanes d*, *h*, *l*, and *o*), confirming the specificity of the binding reactions. The same experiment was performed with extracts from all four stages of the tumorigenesis pathway, and as expected from previous findings (22), GR binds to these sequences at all cell stages with similar efficiency (data not shown). Furthermore, binding of GR to the labeled consensus GRE (Fig. 6*B*, *lane b*) is disrupted by 50-fold excess of the unlabeled putative P1 (*lane c*), P2 (*lane d*), and P3 (*lane e*), which is as efficient as using the same molar excess of unlabeled consensus GRE (S) (*lane f*). In the reverse experiment, binding to all labeled putative GREs, P1, P2, and P3 (Fig. 6*C*, *lanes b, e*, and *h*), could be abolished by competition with unlabeled consensus GRE (S) (*lanes c, f*, and *i*). In summary, these experiments demonstrate that GR can specifically bind *in vitro* to non-consensus GRE sequences in the Bcl-x promoter.

To determine whether GR interacts directly with this region of the Bcl-x promoter *in vivo*, we used the chromatin immunoprecipitation assay. Using an antibody raised against GR to immunoprecipitate sequences bound by GR, we observed increased occupancy by GR at the Bcl-x promoter after addition of dexamethasone (Fig. 7, compare *lanes 7* and *8*), whereas no signal could be amplified from a fragment corresponding to a control element from the Bak promoter, which lacks functional GREs. These observations were dependent on the use of an antibody raised against GR to immunoprecipitate efficiently the sequences of the Bcl-x promoter containing putative GREs, whereas a control antibody (also rabbit serum, against cyclin A) failed to recruit either of the promoter sequences in a ligand-dependent manner (*lanes 5* and *6*). Thus, GR binds to the functional GREs sequences identified in the Bcl-x promoter *in vivo*.

Exogenous Expression of Bcl-x_L Reduces Apoptosis in FS Cells—Apoptosis is reduced by dexamethasone in FS cells, and this correlates with transcriptional activation of the Bcl-x promoter and increased expression of the anti-apoptotic protein, Bcl-x_L. To determine whether enhanced Bcl-x_L expression is

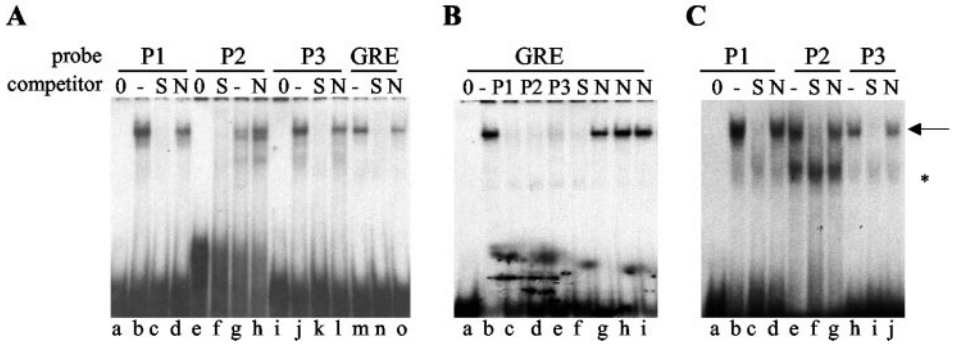

FIG. 6. **Gel retardation assays.** *in vitro* DNA binding of GR from nuclear extracts of dexamethasone-treated FS cells. *A*, the various ^{32}P end-labeled oligonucleotides used as probes (*P1–P3*) are indicated at the *top*. Competition reactions were performed using 50-fold excess of the corresponding unlabeled oligonucleotide (specific (same as labeled) probe (*S*)) (*lanes c*, *f*, *k*, and *n*) or 200-fold excess of a nonspecific oligonucleotide (*N*) (*lanes d*, *h*, *l*, and *o*). *B*, a ^{32}P end-labeled consensus GRE-TAT oligonucleotide used as probe. Competition reactions with 50-fold excess of unlabeled oligonucleotides (*P1–3*, *lanes c–e*, respectively, as well as *GRE*, *S*, *lane f*) or nonspecific oligonucleotides (*N*, *lanes g–i*). *C*, labeled probes are indicated at the *top*). Competition reactions were performed with unlabeled GRE (*S*, *lanes c*, *f*, and *i*) or nonspecific oligonucleotide (*N*, *lanes d*, *g*, and *j*). The *asterisk* indicates the position of a nonspecific complex. *Lane a* (in *A–C*), and *lanes e* and *i* (in *A*) represent incubation in the absence of extract.

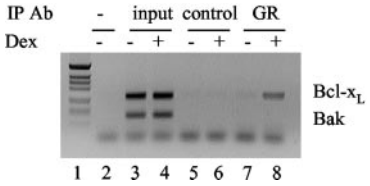

FIG. 7. **ChIP assay.** Cells were untreated or were incubated for 1 h with dexamethasone (*Dex*). Binding of GR to the Bcl-x promoter in FS cells was determined *in vivo* with the ChIP assay (*lanes 7* and *8*). As controls, sample lysates were also incubated with an antibody against cyclin A (*lanes 5* and *6*) or without antibody (*lane 2*), and PCR amplifications included primers designed to detect a control segment from the endogenous Bak promoter that lacks functional GREs. *Lane 1* is the molecular weight marker; *lanes 3* and *4* represent input signals obtained from 0.5% input chromatin, whereas ~3% of the immunoprecipitated material was amplified. *IP Ab*, immunoprecipitation antibody.

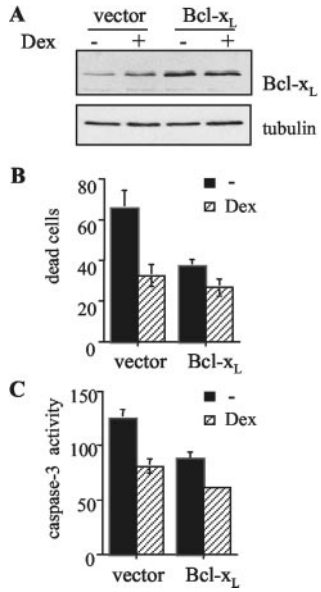

FIG. 8. **Over-expression of Bcl-x_L in FS cells.** *A*, FS cells were transfected with a Bcl-x_L expression vector or control vector and treated with 100 nM dexamethasone (*Dex*) for 48 h. Whole cell extracts were analyzed for Bcl-x_L protein expression. *B*, number of dead cells in Bcl-x_L-transfected FS cell cultures. Values represent the mean of 12 independent counts ($\times 10^4$). *C*, caspase-3 enzyme activity in Bcl-x_L-transfected FS cells. Caspase activity is given in absorbance units ($\times 10^{-3}$).

sufficient to reduce apoptosis in FS cells, we transfected an expression vector encoding Bcl-x_L, driven by the cytomegalovirus promoter, into FS cells in the absence or presence of dexamethasone. Transfection of this construct generated a level of Bcl-x_L protein expression similar to or higher than that obtained by hormone treatment in FS cells transfected with the cytomegalovirus vector alone (Fig. 8*A*). Dexamethasone did not increase the level of Bcl-x_L further. Determination of cell death upon transfection with the Bcl-x_L expression vector alone indicated that the number of dead cells (Fig. 7*B*) and caspase-3 activity (Fig. 8*C*) were reduced significantly ($p < 0.005$) compared with that achieved by dexamethasone treatment of FS cells transfected with vector alone (or untransfected; data not shown). Importantly, Bcl-x_L overexpression in the absence of hormone had a similar effect in both assays, demonstrating that Bcl-x_L inhibits apoptosis in FS cells. The number of dead cells (Fig. 8*B*) and caspase-3 activity (Fig. 8*C*) were further reduced upon dexamethasone treatment ($p < 0.005$) despite apparently constant levels of Bcl-x_L protein.

DISCUSSION

Apoptosis has been found to be widespread in many tumors (36, 37) and yet limited in others (38). GR can display either pro-apoptotic or anti-apoptotic activity depending on cell context (39, 40). We show here that in the absence of glucocorticoids apoptosis increases during fibrosarcoma development, whereas in the presence of dexamethasone it is reduced to those levels found in the earlier stages of the tumorigenic pathway.

Few studies of caspase activity during tumor development have been reported. Caspase-3-like activity has been observed to increase during the development of colorectal carcinoma (41), thus representing a situation similar to that found in the absence of glucocorticoids during fibrosarcoma development. Although involved in some forms of apoptosis, caspase-1 also promotes inflammatory responses by regulating cytokine signaling (42). Increasing caspase-1 activity during fibrosarcoma development may therefore have implications not only for apoptosis but also for inflammation.

Our results demonstrate that decreasing Bcl-x_L expression results primarily from decreasing *Bcl-x* transcription. This correlates inversely with increasing transcription of the BPV-1 genome and E5, E6, and E7 oncogene expression in MF, AF, and FS cells (21, 53). Both BPV-1 E6 and E7 oncoproteins sensitize cells to apoptosis induced by tumor necrosis factor-α (43); thus it can be speculated that these proteins may inhibit Bcl-x transcription. Notably, the angiogenic growth factor bFGF increases Bcl-x expression in epithelial cells (44). Be-

cause the expression of bFGF becomes increasingly extracellular at the AF stage of fibrosarcoma development (45), it is possible that decreasing levels of intracellular bFGF contribute to decreasing Bcl-x_L expression in AF and FS cells. In addition, the transcription factor AP-1 has been proposed to inhibit Bcl-x_L expression (46), and therefore the increased expression and activity of the AP-1 component proteins c-Jun and JunB in AF and FS cells (22, 47) may also reduce Bcl-x_L expression.

Increased Bcl-x_L mRNA expression following glucocorticoid treatment has been observed in human gastric cancer cells (6), where enhanced Bcl-x_L mRNA stability was found to represent a part of the mechanism underlying the protective effect of dexamethasone. However, dexamethasone does not affect the stability of Bcl-x_L mRNA in fibrosarcoma cells (53). The reported increased expression of Bcl-x_L mRNA in human myeloid leukemic cells required 24 h of hormone treatment (48), whereas we clearly detected up-regulation of Bcl-x_L after 2 h of dexamethasone treatment. Subsequent studies by other groups have also demonstrated the importance of increasing Bcl-x_L expression for the inhibition of apoptosis by glucocorticoids in a variety of cell types (3, 8, 31, 49). However, none of these reports identified the mechanism by which GR might control Bcl-x expression.

Further extending the initial observations of these studies, we have demonstrated that the up-regulation of Bcl-x_L expression by dexamethasone occurs at the transcriptional level and is increased during fibrosarcoma development. The increased expression of Bcl-x_L is most likely achieved by specific binding of GR to the identified GRE-like sequences within the Bcl-x promoter. In fact, these sequences also confer hormone responsiveness to a heterologous promoter in COS-7 cells, indicating that the presence of ligand-activated GR is sufficient to induce Bcl-x transcription in other cell types. Furthermore, specific activation of the Bcl-x promoter by dexamethasone requires the cooperation of the various GREs identified, because deletion or mutation of these elements results in a reduction in responsiveness. This observation supports the prediction made by Nordeen and colleagues (35) that most natural response elements are suboptimal elements and that cooperativity among these individually weak sites contributes to the inducibility of the promoter. Because Bcl-x expression is higher in untreated NF than in untreated FS cells, one possibility is that the Bcl-x promoter is repressed in FS cells and that dexamethasone treatment results in de-repression. Although this may be the case, the reduced expression of Bcl-x in FS cells is unlikely to involve GR binding to GREs in the Bcl-x promoter, because their mutation does not affect transcription in the absence of hormone.

Importantly, Bcl-x_L is responsible for the ability of dexamethasone to inhibit apoptosis in fibrosarcoma cells, as shown by ectopic expression of Bcl-x_L. As expression of Bcl-x_L decreases during fibrosarcoma development in the absence of glucocorticoids, increasing up-regulation of Bcl-x_L expression by dexamethasone serves to maintain Bcl-x_L levels during tumor development, and thus apoptosis remains approximately constant. In effect, Bcl-x_L expression becomes increasingly dependent on glucocorticoids during fibrosarcoma development. Increased Bcl-x_L expression and reduced apoptosis correlate in FS cells with decreased caspase-3, but not caspase-1, activity in response to glucocorticoids. Such a correlation has also been reported by Messmer *et al.* (49). Furthermore, levels of apoptosis in hormone-treated FS cells are comparable with those in earlier cell types, despite the presence of higher caspase-1 activity. These data indicate that caspase-3 is likely to play a more important role than caspase-1 in determining apoptosis in FS cells.

It may be noteworthy that we also observed dexamethasone regulation of Bcl-x expression in both normal and transformed human mammary epithelial cells. Apoptosis of immortalized mouse mammary epithelial cells can be decreased by dexamethasone treatment, associated with increased Bcl-x mRNA expression, within 2 h of hormone treatment (8). Intriguingly, an increase in Bcl-x_L protein expression following dexamethasone treatment was readily detectable in transformed SK-BR-3 breast cancer cells but was lower in normal epithelial cells (data not shown). This represents a parallel situation to that observed during fibrosarcoma progression, where the ability of hormone to induce Bcl-x_L expression increases during tumor development; it may reflect the ability of normal cells to limit, or of tumor cells to enhance, regulation of gene expression by specific steroid hormone receptors as has been suggested previously (22). Such a phenomenon may be a result of increased expression of steroid hormone receptor co-factors during tumor development, and indeed co-activators such as E6-AP and AIB1 are overexpressed in certain tumor types (50, 51).

It is interesting to consider that the discovery of regulation of Bcl-x_L expression by glucocorticoids during tumorigenesis may have clinical relevance. Glucocorticoids such as dexamethasone are used as anti-emetics in the treatment of several cancers. One study has identified a strong negative correlation between Bcl-x_L expression and sensitivity to a wide variety of cytotoxic agents in 60 cancer cell lines (18). Our results suggest that Bcl-x_L expression (and thus cell survival) may be promoted by dexamethasone. Therefore, glucocorticoid treatment of tumors may reduce the cytotoxic effects of chemotherapy on tumor cells and may be contraindicated in those tumors with highly inducible Bcl-x_L expression. A similar situation may exist in malignant gliomas, where glucocorticoids have been reported to interfere with chemotherapy response (52). Importantly, indications that expression of Bcl-x_L may be more glucocorticoid-dependent in tumor cells than in normal cells suggest that glucocorticoid therapy could have a certain degree of tumor specificity.

The enhanced expression of Bcl-x_L by GR during fibrosarcoma development reflects the unexpected transition in the transcriptional activity of GR and reveals an alternative mode of regulation for steroid hormone receptors. In summary, increasing GR transcriptional activity is likely to exert a protective effect by enabling the reduction of apoptosis at the tumor stage of fibrosarcoma development and, therefore, prolonging tumor cell survival.

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Glucocorticoids Inhibit Apoptosis during Fibrosarcoma Development by Transcriptionally Activating Bcl-x$_L$

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